DESCRIPTION

POLYNUCLEOTIDE ENCODING OXALATE DECARBOXYLASE FROM *ASPERGILLUS NIGER* AND METHODS OF USE

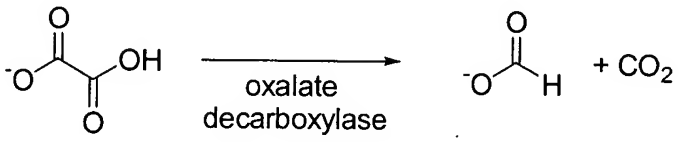
The subject invention was made with government support under a research project supported by National Institutes of Health Grant No. DK53556.

Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application Serial No. 60/404,892, filed August 20, 2002.

Background of the Invention

Oxalic acid, a compound that is toxic to almost all organisms (Hodgkinson, 1977), plays several important roles in fungal growth and metabolism (Dutton *et al.*, 1996), and in biological mechanisms underlying fungal pathogenesis. For example, *Aspergillus niger*, which can colonize lung tissue in immunocompromised individuals, excretes enough oxalate to form crystalline calcium salts as part of necrotizing otomycosis (Landry *et al.*, 1993) and, in certain cases, can give rise to fatal pulmonary oxalosis (Kimmerling *et al.*, 1992; Metzger *et al.*, 1984). A number of enzymes have evolved in plants (oxalate oxidase) (Kotsira *et al.*, 1997), fungi (oxalate decarboxylase) (Lillehoj *et al.*, 1965) and bacteria (oxalyl-CoA decarboxylase) (Quayle, 1963) to remove oxalate from the environment. Oxalate decarboxylase (OxDC) catalyzes a remarkable transformation in which the C-C bond in oxalate is cleaved to give carbon dioxide and formate:

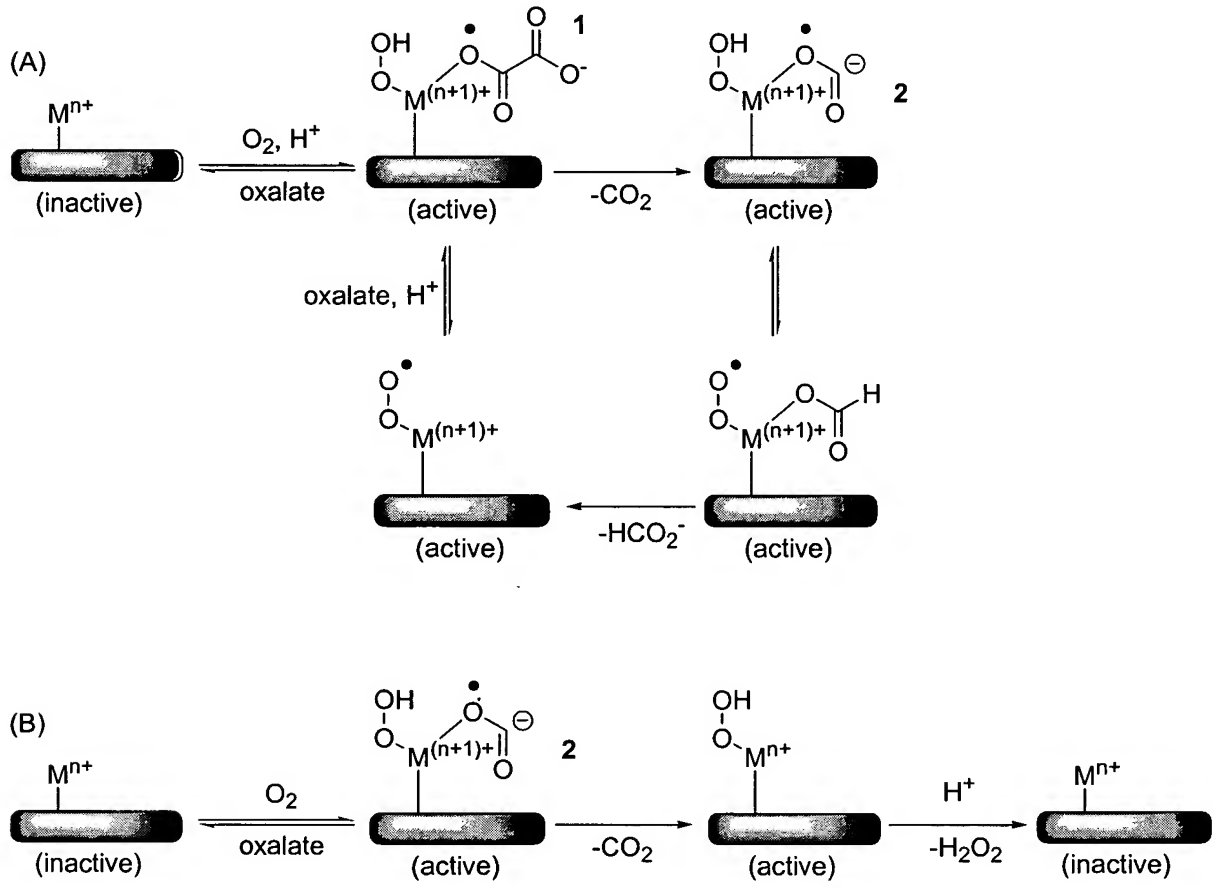


The enzyme is presumably important in fungal metabolism as a protective agent against internalization of neutral oxalate formed in the environment as the soil pH drops due to wood degradation or secretion of oxalic acid. Oxalate decarboxylase was first isolated from

basidiomycete fungi (Shimazono, 1955), and has subsequently been identified in several species of filamentous fungi, including *Myrothecium verrucaria* (Lillehoj *et al.*, 1965), certain strains of *Aspergillus niger* (Emiliani *et al.*, 1964) and *Flammulina velutipes* (Mehta *et al.*, 1991), and the common button mushroom *Agaricus bisporus* (Kathiara *et al.*, 2000). OxDC expression can also be induced in the white-rot fungus *Coriolus versicolor* (Shimazono *et al.*, 1957), and very recent work has also shown that OxDC is present in *Bacillus subtilis* (Tanner *et al.*, 2000), although this appears to be the only bacterium in which the presence of this enzyme has been unambiguously demonstrated. While it has been demonstrated that the bacterial OxDC is manganese-dependent (Tanner *et al.*, 2001), the detailed catalytic mechanism by which oxalate is converted to formate and carbon dioxide has not yet been elucidated.

Early experiments employing the *Aspergillus niger* OxDC showed that (i) enzymatic CO_2 evolution requires oxalate to the exclusion of other biologically relevant carboxylic acids, (ii) oxygen is required for catalytic turnover, although high oxygen tensions inhibit the enzyme (Emiliani *et al.*, 1968), and (iii) a sub-stoichiometric quantity of oxygen is converted to hydrogen peroxide during the reaction. Weak reductants such as phenylenediamines and diphenols activate the enzyme, whereas treatment with strong reductants such as dithionite and hydroxylamine eliminate OxDC activity. No evidence was found for the presence of exogenous cofactors in the native *Aspergillus niger* OxDC, and the enzyme was reported not to contain iron and copper ions as purified. A general, oxygen-dependent, mechanism involving the formation of free radical species was proposed to account for these experimental observations (Emiliani *et al.*, 1968). In light of the demonstrated dependence of OxDC activity on dioxygen (Tanner *et al.*, 2001), a hypothetical catalytic mechanism is currently favored in which bound manganese undergoes an oxidation to give a species capable of abstracting an electron directly from oxalate to give the radical anion **1** (Scheme 1A). It is also likely that oxalate binding to manganese precedes that of dioxygen. C-C bond cleavage, which might be expected to be a fast chemical step, then yields **2** and concomitant proton and electron transfer (Su *et al.*, 1998) to give formate regenerates the oxidized metal species. No evidence for the involvement of a redox-active co-factor (Halcrow, 2001) is provided by the recent crystal structure of the bacterial OxDC (Anand *et al.*, 2002). The structures of the fungal and bacterial oxalate decarboxylases are likely to be very similar on the basis of sequence identity and the likely evolutionary relationship between

the two enzymes (Dunwell *et al.*, 2000). The observed correlation between H_2O_2 formation and pO_2 in the OxDC-catalyzed reaction (Emiliani *et al.*, 1968) is consistent with such a mechanism if oxidation of the formyl radical anion **2** takes place to generate CO_2, peroxide anion and an inactive form of OxDC (Scheme 1B).



Scheme 1. (A) Hypothetical mechanism for OxDC-catalyzed conversion of oxalate into CO_2 and formate via homolytic C-C bond cleavage. Note that the interaction of the manganese ion (M^{n+}) with oxygen and oxalate remains to be established experimentally. (B) Side reaction proposed to consume oxygen during turnover.

Although it has been speculated that Mn(III) and Mn(IV) are the redox active forms of the metal during catalysis (Anand *et al.*, 2002), there is no published evidence to support such a claim. Equally, the intermediacy of a protein-based radical cannot be ruled out on the basis of current biochemical and structural information on *Bacillus subtilis* OxDC. This proposal has the merit of rationalizing the observed correlation between the amounts of hydrogen peroxide

formed under the assay conditions and the partial pressure of oxygen. Chemical precedent for a mechanism involving radical-dependent decarboxylation of oxalate has been obtained in model chemical studies (Drummond *et al.*, 1953; Halliwell, 1972), including direct electron-nuclear double resonance (ENDOR) observation of formate radical produced by irradiation of oxalate crystals (Edlund *et al.*, 1973). Additional support is provided by the Kolbe reaction in which one-electron electrochemical oxidation of carboxylic acids results in production of CO_2 and daughter radicals (Bard *et al.*, 1978). Nevertheless, a radical-based mechanism for OxDC-catalyzed oxalate degradation would gain considerable credence upon observation of paramagnetic species formed on incubation of the enzyme with substrate.

Kidney-urinary tract stone disease (urolithiasis) is a major health problem throughout the world. Most of the stones associated with urolithiasis are composed of calcium oxalate alone or calcium oxalate plus calcium phosphate. Other disease states have also been associated with excess oxalate. These include, vulvodynia, oxalosis associated with end-stage renal disease, cardiac conductance disorders, Crohn's disease, and other enteric disease states.

Oxalic acid (and/or its salt-oxalate) is found in a wide diversity of foods, and is therefore, a component of many constituents in human and animal diets. Increased oxalate absorption may occur after foods containing elevated amounts of oxalic acid are eaten. Foods such as spinach and rhubarb are well known to contain high amounts of oxalate, but a multitude of other foods and beverages also contain oxalate. Because oxalate is found in such a wide variety of foods, diets that are low in oxalate and which are also palatable are hard to formulate. In addition, compliance with a low oxalate diet is often problematic.

Normal tissue enzymes also produce endogenous oxalate metabolically. Oxalate (dietary oxalate that is absorbed as well as oxalate that is produced metabolically) is not further metabolized by tissue enzymes and must therefore be excreted. This excretion occurs mainly via the kidneys. The concentration of oxalate in kidney fluids is critical, with increased oxalate concentrations causing increased risk for the formation of calcium oxalate crystals and thus the subsequent formation of kidney stones.

The risk for formation of kidney stones revolves around a number of factors that are not yet completely understood. Kidney-urinary tract stone disease occurs in as much as 12% of the population in Western countries and about 70% of these stones are composed of calcium oxalate

or of calcium oxalate plus calcium phosphate. Some individuals (*e.g.*, patients with intestinal disease such as Crohn's disease, inflammatory bowel disease, or steatorrhea and also patients that have undergone jejunoileal bypass surgery) absorb more of the oxalate in their diets than do others. For these individuals, the incidence of oxalate urolithiasis increases markedly. The increased disease incidence is due to increased levels of oxalate in kidneys and urine, and this, the most common hyperoxaluric syndrome in man, is known as enteric hyperoxaluria. Oxalate is also a problem in patients with end-stage renal disease and there is recent evidence (Solomons *et al*, 1991) that elevated urinary oxalate is also involved in vulvar vestibulitis (vulvodynia).

Bacteria that degrade oxalate have been isolated from human feces (Allison *et al.*, 1986). These bacteria were found to be similar to oxalate-degrading bacteria that had been isolated from the intestinal contents of a number of species of animals (Dawson *et al.*, 1980; Allison *et al.*, 1981; Daniel *et al.*, 1987). These bacteria are different from any previously described organism and have been given both a new species and a new genus name (Allison *et al.*, 1985).

Not all humans carry populations of *O. formigenes* in their intestinal tracts (Allison *et al.*, 1995; Doane *et al.*, 1989). There are low concentrations or a complete lack of oxalate degrading bacteria in the fecal samples of persons who have had jejunoileal bypass surgery (Allison *et al.*, 1986). Also, certain humans and animals may maintain colonies of *O. formigenes* but nevertheless have excess levels of oxalate for reasons that are not clearly understood.

U.S. Patent No. 6,355,242 and published international patent application WO 98/52586 disclose delivery of bacteria and/or oxalate-degrading enzymes to intestinal tracts of persons or animals, thereby reducing oxalate in the intestinal tract of those persons or animals who are at risk for oxalate related disease.

OxDC of *Aspergillus niger*, which converts oxalate directly to formate and carbon dioxide without the need for exogenous co-factors, can provide a therapeutic approach at a significant reduction in cost. A second benefit of using *Aspergillus niger* OxDC is that the enzyme has a pH-optimum of 4.2, making it useful for oxalate degradation in the upper intestine. Since *Aspergillus niger* is also used in the production of citrate, which is then added to food products and dietary supplements, it is likely that no significant side effects will be observed when this form of OxDC is administered in the human gastrointestinal tract.

Brief Summary of the Invention

The subject invention pertains to polynucleotides encoding the enzyme oxalate decarboxylase from the filamentous fungus *Aspergillus niger* and methods of use. The polynucleotides can be used to express oxalate *Aspergillus niger* decarboxylase that can be used to degrade oxalate for therapeutic and other purposes. The subject invention also pertains to cells and microbes, such as bacteria, which are transformed with a polynucleotide of the present invention encoding an oxalate decarboxylase enzyme.

The subject invention also pertains to plants that are transformed with a polynucleotide of the present invention encoding an oxalate decarboxylase enzyme. Transformed plants of the present invention expressing oxalate decarboxylase can be administered to a human or animal as a constituent of a meal, for example, as a salad or vegetable. In addition, the transformed plant of the present invention can be administered to an animal as a constituent of feed or the plant can be grown in a pasture in which animals are allowed to graze and feed upon the plant.

The subject invention also concerns the use of *Aspergillus niger* oxalate decarboxylase, or a microbe transformed with a polynucleotide of the invention to express oxalate decarboxylase of the invention, to achieve therapeutic oxalate degradation in a human or animal.

The subject invention also pertains to use of oxalate decarboxylase of the invention to degrade oxalate present in fluids, such as blood and urine. For example, oxalate decarboxylase of the invention can be coated or attached to a surface, for example, that of a catheter or other medical device, that might come into contact with a fluid containing oxalate. The attached enzyme can prevent oxalate accumulation or encrustation on those surfaces of a device that are in contact with the fluid.

Brief Description of Drawings

Figure 1 shows an SDS-PAGE gel of oxalate decarboxylase purification fractions obtained from the procedures described in "Materials and Methods" section herein. Lanes from left to right are crude extract, methanol re-suspension, Q-Sepharose fractions and phenyl Sepharose fractions.

Figure 2 shows the deduced primary structure of *Aspergillus niger* oxalate decarboxylase protein from the cDNA sequence shown in Figure 3. Amino acids that define the signal peptide of the protein are shown in italic font. Standard one letter code is used to represent amino acids.

Figure 3A-C shows the alignment of the nucleotide sequences of the gene encoding oxalate decarboxylase *Aspergillus niger* (genomic) OxDC and the cDNA obtained from mRNA isolated from the fungus (cDNA). Underlined residues in the genomic sequence indicate the location of the two introns deduced to be present in the gene by comparison of the sequences. These are both flanked by canonical sequences shown in bold typeface. The TAG sequence at the 3'-end of the gene, also shown in bold typeface, indicates the end of the region coding for the protein product.

Figure 4 shows the DNA sequence encoding oxalate decarboxylase as cloned from genomic DNA of *Aspergillus niger*.

Figure 5 shows the deduced primary structure of *Bacillus subtilis yvrk* protein. Standard one letter code is used to represent amino acids.

Brief Description of Sequences

SEQ ID NO. 1 is a genomic polynucleotide of *Aspergillus niger* encoding an oxalate decarboxylase enzyme that can be used according to the present invention.

SEQ ID NO. 2 is a cDNA sequence of *Aspergillus niger* encoding an oxalate decarboxylase enzyme that can be used according to the present invention.

SEQ ID NO. 3 is the amino acid sequence of an oxalate decarboxylase enzyme of *Aspergillus niger* encoded by SEQ ID NO. 1.

SEQ ID NO. 4 is an amino acid sequence of an oxalate decarboxylase enzyme of the invention with the amino acid leader sequence removed.

SEQ ID NO. 5 is a sequence of a PCR primer that can be used according to the present invention.

SEQ ID NO. 6 is a sequence of a PCR primer that can be used according to the present invention.

SEQ ID NO. 7 is a partial sequence of the oxalate decarboxylase enzyme of the present invention.

SEQ ID NO. 8 is a predicted partial sequence of the oxalate decarboxylase enzyme of the present invention.

SEQ ID NO. 9 is the deduced primary structure of *Bacillus subtilis yvrk* protein according to the present invention.

Detailed Disclosure of the Invention

The subject invention concerns polynucleotides encoding the enzyme oxalate decarboxylase from the filamentous fungus *Aspergillus niger*. The amino acid sequence of the oxalate decarboxylase enzyme from *Aspergillus niger* is shown in Figure 2 (SEQ ID NO. 3). The subject invention pertains to the enzyme having the sequence shown in Figure 2 (SEQ ID NO. 3), as well as the enzyme lacking the leader sequence (shown in italics in Figure 2), *i.e.*, a polypeptide of SEQ ID NO 4. A cDNA sequence that encodes the oxalate decarboxylase enzyme from *Aspergillus niger* is shown in the bottom row of nucleotide sequence in Figure 3 (SEQ ID NO. 2). The genomic sequence from *Aspergillus niger* encoding oxalate decarboxylase is shown in the top row of nucleotide sequence in Figure 3 (SEQ ID NO. 1). The subject invention also concerns the polypeptides of the invention complexed with a metal. Preferably, the metal is manganese, iron, or copper. More preferably, the metal is manganese.

The subject invention also concerns pharmaceutical and nutraceutical compositions for the introduction of the oxalate decarboxylase of the present invention and/or bacteria or other cells that have been transformed with a polynucleotide of the present invention into the intestine of a human or animal. In one embodiment, the transformed bacteria or cell or enzyme has been lyophilized or frozen. A liquid or paste form can be encapsulated in a gel capsule or provided with other forms of enteric protection. Preferably, the gel capsule material or the material providing enteric protection is resistant to degradation by the acidity and enzymes of the stomach but can be degraded, with concomitant release of the enzyme and/or transformed bacteria or cell of the invention, by the higher pH and bile acid contents present in the human or animal intestinal tract.

The subject invention also concerns transgenic animals in which a polynucleotide encoding an oxalate decarboxylase of the invention has been incorporated into the animal's genome. Methods for preparing transgenic animals are well known in the art.

The subject invention also concerns an enzyme delivery system comprising a plant which has been transformed with a polynucleotide of the subject invention encoding oxalate decarboxylase, which when expressed can degrade oxalate. Transformed plants of the present invention expressing oxalate decarboxylase can be administered to a human or animal as a constituent of a meal, for example, as a salad or vegetable. In addition, the transformed plant of the present invention can be administered to an animal as a constituent of feed or the plant can be grown in a pasture in which animals are allowed to graze and feed upon the plant.

The subject application also concerns plants transformed with polynucleotides of the present invention that encode oxalate decarboxylase from *Aspergillus niger*. Plants that can be transformed with the subject polynucleotide include both monocotyledonous and dicotyledonous plants. Plants within the scope of the present invention include monocotyledonous plants, such as rice, wheat, barley, oats, sorghum, maize, lilies, and millet, and dicotyledonous plants, such as peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce. In a particularly preferred embodiment, the plant is a cereal. Cereals to which this invention applies include, for example, maize, wheat, rice, barley, oats, rye, and millet.

The subject invention also concerns methods for degrading oxalate present in fluids, such as blood and urine, using oxalate decarboxylase of the present invention. The subject invention can be used to prevent or minimize encrustation of oxalate crystals on any device, such as a catheter, that comes into contact with oxalate-containing fluids. An oxalate decarboxylase enzyme of the invention can be provided on or in any devices that come into contact with fluids that contain or may contain oxalate. For example, the enzyme can be coated to or attached on the inside of a medical catheter or stent. The enzyme could also be provided in dialysis cartridges to degrade oxalate present in a patient's biological fluid.

The subject invention also concerns methods and compositions for assaying for the presence of oxalate. In one embodiment, the method comprises contacting a sample to be assayed with an oxalate decarboxylase of the present invention and then determining the presence of either carbon dioxide or formate generated from the reaction of the enzyme with oxalate.

The subject invention also concerns pharmaceutical and nutraceutical compositions for the introduction of the oxalate decarboxylase of *Bacillus subtilis* and/or bacteria or other cells that have been transformed with a polynucleotide encoding the oxalate decarboxylase of *Bacillus subtilis* into the intestine of a human or animal. In one embodiment, the transformed bacteria or cell or enzyme has been lyophilized or frozen. A liquid or paste form can be encapsulated in a gel capsule or provided with other forms of enteric protection. Preferably, the gel capsule material or the material providing enteric protection is resistant to degradation by the acidity and enzymes of the stomach but can be degraded, with concomitant release of the enzyme and/or transformed bacteria or cell of the invention, by the higher pH and bile acid contents present in the human or animal intestinal tract.

The subject invention also concerns transgenic animals in which a polynucleotide encoding oxalate decarboxylase of *Bacillus subtilis* has been incorporated into the animal's genome. Methods for preparing transgenic animals are well known in the art.

The subject invention also concerns an enzyme delivery system comprising a plant which has been transformed with a polynucleotide encoding oxalate decarboxylase of *Bacillus subtilis*, which when expressed can degrade oxalate. Transformed plants of the present invention expressing oxalate decarboxylase of *Bacillus subtilis* can be administered to a human or animal as a constituent of a meal, for example, as a salad or vegetable. In addition, the transformed plant of the present invention can be administered to an animal as a constituent of feed or the plant can be grown in a pasture in which animals are allowed to graze and feed upon the plant.

The subject application also concerns plants transformed with polynucleotides encoding the oxalate decarboxylase of *Bacillus subtilis*. Plants that can be transformed with the subject polynucleotide include both monocotyledonous and dicotyledonous plants. Plants within the scope of the present invention include monocotyledonous plants, such as rice, wheat, barley, oats, sorghum, maize, lilies, and millet, and dicotyledonous plants, such as peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce. In a particularly preferred embodiment, the plant is a cereal. Cereals to which this invention applies include, for example, maize, wheat, rice, barley, oats, rye, and millet.

The subject invention also concerns methods for degrading oxalate present in fluids, such as blood and urine, using oxalate decarboxylase of *Bacillus subtilis*. The subject invention can be used to prevent or minimize encrustation of oxalate crystals on any device, such as a catheter, that comes into contact with biological fluids. Oxalate decarboxylase of *Bacillus subtilis* can be provided on or in any devices that come into contact with fluids that contain or may contain oxalate. For example, the enzyme can be coated to or attached on the inside of a medical catheter or stent. The enzyme could also be provided in dialysis cartridges to degrade oxalate present in a patient's biological fluid.

The methods and compositions of the present invention can be used with humans and other animals. The other animals contemplated within the scope of the invention include domesticated, agricultural, or zoo- or circus-maintained animals. Domesticated animals include, for example, dogs, cats, rabbits, ferrets, guinea pigs, hamsters, pigs, monkeys or other primates, and gerbils. Agricultural animals include, for example, horses, mules, donkeys, burros, cattle, cows, pigs, sheep, and alligators. Zoo- or circus-maintained animals include, for example, lions, tigers, bears, camels, giraffes, hippopotamuses, and rhinoceroses.

Polynucleotides of the present invention can be composed of either RNA or DNA. Preferably, the polynucleotides are composed of DNA. The subject invention also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode oxalate decarboxylase enzymes disclosed herein. In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, polypeptides of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences that encode amino acid substitutions, deletions, additions, or insertions, which do not materially alter the functional activity of the polypeptide, encoded by the polynucleotides of the present invention.

Substitution of amino acids other than those specifically exemplified in the sequence of oxalate decarboxylase disclosed herein is also contemplated within the scope of the present invention. Amino acids can be placed in the following classes: non-polar, uncharged polar,

basic, and acidic. Conservative substitutions whereby an oxalate decarboxylase polypeptide having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the oxalate decarboxylase having the substitution still retains substantially the same activity as wild type polypeptide. Table 1 below provides a listing of examples of amino acids belonging to each class.

Table 1.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

Also contemplated are substitutions of naturally occurring amino acids in the oxalate decarboxylase sequence with non-natural amino acids. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ-amino butyric acid, ε-amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ-butylglycine, τ-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form.

The scope of the invention also includes amino acid substitutions in the sequence of the polypeptide that change the pH optimum at which the polypeptide exhibits the highest level of enzymatic activity. Techniques for making such amino acid substitutions and assaying the

polypeptide for pH optimum are well known in the art (Neves-Peterson *et al.*, 2001; Nielson *et al.*, 1999; Shaw *et al.*, 1999).

Polynucleotides and proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also concerns polynucleotides, which encode fragments of a full-length oxalate decarboxylase enzyme of the invention, so long as those fragments retain substantially the same functional activity as full-length polypeptide. The fragments of an oxalate decarboxylase polypeptide encoded by these polynucleotides are also within the scope of the present invention. Fragments of the full-length sequence can be prepared using standard techniques known in the art.

The subject invention also contemplates those polynucleotide molecules encoding oxalate decarboxylase enzymes having sequences that are sufficiently homologous with the wild type sequence of *Aspergillus niger* so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis *et al.*, 1982). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25 C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x

Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.*, 1983):

Tm=81.5 C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Materials and Methods

Culture Conditions. *Aspergillus niger* (ATCC 26550) was maintained on potato dextrose agar plates at 4 °C. Inoculating a starter culture of ATCC medium 950 with a loopful of Aspergillus niger spores produced mycelium for OxDC purification. The literature procedures for inducing OxDC production were modified by the substitution of sucrose for glucose (by weight), NH_4Cl for $NaNO_3$ (by molarity) and the addition of 10 mM sodium oxalate (Sigma). After growth at 30 °C for several days, this starter culture was used to inoculate larger cultures. Fungus for DNA isolation was grown on yeast extract-peptone-dextrose medium (ATCC medium 1005), with cultures being shaken at 37 °C until the mycelium was confluent. Mycelium was harvested by vacuum filtration, washed with de-ionized water, frozen in powdered dry ice, lyophilized, and stored at –80 °C until used in subsequent experiments.

Isolation of Native OxDC. Freeze-dried mycelium was ground in a mortar and pestle with powdered dry ice. After sublimation, the powder was suspended in 20 mM NaOAc pH 5.6 buffer plus 0.1% Tween 20. Insoluble material was removed by centrifugation, and methanol added to the supernatant to a final concentration of 50% v/v. The resulting mixture was incubated at 0 °C for 30 minutes before collection of precipitated material by centrifugation. The

pellet was re-suspended in 20 mM NaOAc pH 5.6 ("purification buffer") and incubated overnight at 4 °C. Insoluble material was again removed by centrifugation, and the supernatant applied to a 1.6 × 12.5 cm Q-Sepharose Hi-Performance column (Amersham Pharmacia Biotech). The column was washed with purification buffer, and then eluted with a 0-1 M NaCl gradient. Active fractions were pooled, before the addition of solid $(NH_4)_2SO_4$ to a final concentration of 1 M. After re-suspension and loading onto a 1.6 × 12 cm Phenyl Sepharose Hi-Performance column, bound protein was washed with purification buffer containing 1 M $(NH_4)_2SO_4$, then eluted with a 1-0 M ammonium sulfate gradient. Active fractions were pooled, diafiltered and concentrated, and stored at 4 °C prior to characterization. Additional details of this purification procedure are shown in Table 2.

Table 2. Purification of *Aspergillus niger* oxalate decarboxylase

Fraction	Activity (nmol/min)	Specific Activity (I.U./mg)	Total Activity (I.U.)	Fold-Purification	% Yield
Crude extract	1.4	0.02	51		
Methanol Supernatant	8.8	0.52	88	34	100*
Q Sepharose	5.4	5.7	33	376	37
Phenyl Sepharose	9.2	10	18	662	21

Enzyme Assay. Assays consisted of 50 mM NaOAc pH 5.2, 0.2% Tween 20, 2 mM *o*-phenylenediamine, 30 mM potassium oxalate, and enzyme in a volume of 100 µL. Turnover was initiated by addition of substrate. Mixtures were incubated at ambient temperature (21-22 °C), and then the reaction was quenched by addition of 10 µL 1 N NaOH. The amount of formate produced in the enzyme-catalyzed reaction was measured using a formate dehydrogenase (FDH) assay consisting of 100 mM potassium phosphate pH 7.8, 1.5 mM NAD^+, and 0.1 I.U. FDH (1 mL final volume). Absorbance at 340 nm was measured after incubation at

37 °C for 30 minutes. Formate was quantitated by comparison to a standard curve generated by spiking protein-free OxDC assays with known amounts of sodium formate.

Metal Analysis of Aspergillus niger OxDC. Purified *Aspergillus niger* OxDC was treated to remove adventitious, surface-bound metals by incubating 9 mg/mL OxDC (100 μL) with 10 mM *o*-phenanthroline (10 μL) on ice for 30 minutes. The enzyme was then desalted over G25 Sephadex resin that had been pre-treated with 20 mM NaOAc, pH 5.2, containing 2 mM EDTA and then equilibrated with Chelex-100-treated 20 mM NaOAc buffer, pH 5.2. In these experiments, all glassware was washed with 1 M HNO_3 and rinsed with deionized water (18.3 M) to remove exchangeable metal ions prior to use. Samples of OxDC treated in this manner were then divided into two aliquots for EPR and inductively coupled plasma-atomic emission (ICP-AE) spectroscopy. Metal content was determined by ICP-AE spectroscopy using protein samples made by diluting 100 μL of OxDC (0.5 mg) with 9.9 mL deionized water. All analyses were performed in the Department of Chemistry at the University of Florida. Calculations of the metal content in native *Aspergillus* niger OxDC employed standard procedures (see supplementary material).

Chromosomal DNA Cloning. Freeze-dried mycelium (0.5 g) taken from shake cultures of confluent *Aspergillus niger* was gently ground in liquid N_2, using a mortar and pestle, to give a fine powder. Care was taken during this procedure so as to prevent shearing high-molecular weight DNA by excessive grinding. The resulting powder was extracted with Qiagen "QBT" buffer (20 mL) supplemented with 0.5% v/v Triton X-100, before the addition of solutions of ribonuclease A (100 μL) and 14 mg/mL Proteinase K (100 μL). The extract was incubated for 30 min at ambient temperature, and then for 15 min at 50 °C before being loaded onto a Qiagen Genomic Tip and purified. The resulting high molecular weight DNA was digested thoroughly using *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I restriction enzymes.

Polymerase chain reaction (PCR) primers were designed assuming a close nucleotide sequence relationship between the genes encoding OxDC in *Aspergillus phoenices* (Scelonge *et al.*, 1998) and *Aspergillus niger*. A mutagenic 5'-primer (5'-GTCCTCGAGAAAAGATACCAG-3') (SEQ ID NO. 5) was employed to introduce a *Xho*I site and a proteolytic cleavage site, for use in future expression experiments, immediately upstream of the codon of Tyr-24 in the putative *Aspergillus niger* gene sequence. This primer was

combined with a reverse primer (5'-TCATCTACTCACTTGGGCTCCGAATTG –3') (SEQ ID NO. 6) matching the 3'-end of the gene in *Aspergillus phoenices*. Thirty cycles of amplification were performed (95 °C, 1' denaturation; 45 °C primer annealing, 30 s; 74 °C primer extension, 3') with *Pfu* polymerase (Promega, Madison, WI), and the resulting PCR product purified by phenol:chloroform:isoamyl alcohol extraction, chloroform extraction, and ethanol precipitation. The plasmid pPIC9K was digested with *Sna*BI, treated with alkaline phosphatase, and purified prior to overnight ligation with the purified PCR product. Competent JM109 cells (>10^8 CFU/μg) were transformed, and white colonies screened by *Xho*I digestion of alkaline lysis/miniprepped plasmid DNA. Plasmid that produced two bands upon *Xho*I digestion was purified, and submitted for nucleotide sequencing at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Isolation, Purification, and Assay of Native Oxalate Decarboxylase from *Aspergillus niger*.

Previous studies had shown that the presence of OxDC in the mycelium of *Aspergillus niger* was inversely related to detectable oxalate in the extracellular milieu (Emiliani *et al.*, 1964). This might be rationalized by assuming that there is leakage of protonated oxalic acid back into the fungus when the pH of the culture drops due to excretion of oxalic and citric acids during the early stages of *Aspergillus* growth. As a consequence, OxDC expression is induced so as to reduce oxalate concentrations in the mycelium to a non-toxic level. In light of this hypothesis, literature protocols for inducing OxDC production in *Aspergillus niger* were modified by adding 10 mM sodium oxalate to the minimal media upon which the fungus was grown. Under these conditions, adequate amounts of enzyme could be isolated from the fungal mycelium for the work described here. Previous studies had demonstrated that native *Aspergillus niger* OxDC exhibited notable stability in organic solvents, and so initial purification steps involved precipitation of the enzyme with methanol. Subsequent chromatography using

anion exchange and hydrophobic interaction columns gave OxDC as a single band on SDS-PAGE (Figure 1), with a molecular weight in the range expected based on studies of the enzyme isolated from *Flammulina velutipes* (Kathiara *et al.*, 2000). Purified *Aspergillus niger* OxDC exhibited a specific activity of 10 I.U./mg, as determined from steady-state formate production under initial velocity conditions.

Example 2 - Deduced Primary Structure of *Aspergillus niger* OxDC.

Cloning of both the chromosomal and cDNA copies of the OxDC gene showed that (i) there are only two intron sequences in the *Aspergillus niger* decarboxylase gene (Figure 3A-3C), in contrast to the 17 reported for the cognate gene in *Flammulina velutipes* (Kesarwani *et al.*, 2000), and (ii) the intron sequences in the gene encoding OxDC in *Aspergillus niger* have canonical 5' and 3' borders (GT-AG) in contrast to those reported to be present in the cognate gene in *Aspergillus phoenices* (Scelonge *et al.*, 1998). The protein product encoded by the *yvrk* gene in *Bacillus subtilis* (Kunst *et al.*, 1997) shows some homology to *Aspergillus niger* OxDC with 197 (52%) residues in the bacterial OxDC being identical to those in the fungal enzyme.

More importantly for the catalytic mechanism of oxalate degradation, there are two "His-Trp-His" motifs that are conserved among the bacterial and fungal oxalate decarboxylases. Recent work on recombinant *Bacillus subtilis* OxDC suggests that this enzyme contains Mn(II) in its resting state (Tanner *et al.*, 2001; Anand *et al.*, 2002) consistent with our observations on the native *Aspergillus niger* OxDC. In addition, a similar "His-Ile-His" motif present in oxalate oxidase has been shown to be a manganese-binding site by X-ray crystallography (Woo *et al.*, 2000), suggesting a role for at least one, and possibly two metal ions, in OxDC catalysis (Gane *et al.*, 1998).

Example 3 - Biochemical Characterization of Native *Aspergillus niger* OxDC.

With the successful development of culture conditions and purification procedures to obtain milligram amounts of native *Aspergillus niger* OxDC, the biochemical and spectroscopic properties of the enzyme were investigated. N-terminal sequencing of the purified protein, carried out at the Protein Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (UF), revealed that phenylalanine is the first

residue in the mature enzyme, the initial N-terminal sequence being Phe-Gln-Asp-Lys-Pro-Phe-Thr-Pro-Asp-His-Arg (SEQ ID NO. 7), matching the primary structure deduced from the cloned genes encoding OxDC in both *Aspergillus niger* (*vide infra*) and *Aspergillus phoenices* (Scelonge *et al.*, 1998) and confirming the identity of the isolated protein. It was anticipated that the N-terminal region would be Tyr-Gln-Gln-Asp (SEQ ID NO. 8) on the basis of the primary structure deduced from the cloned gene, and shows that the fungal enzyme is synthesized with a leader peptide that may be important in cellular trafficking (Walter *et al.*, 1994). MALDI-TOF measurements on the purified fungal OxDC indicated that the mass of a single subunit of the enzyme is 48,700-48,800 Da, which is consistent with that calculated for the deduced amino acid sequence with the observed N-terminal residue, assuming that the protein contains metal ions and is glycosylated. While the bacterial OxDC has been shown to exist as a hexamer consisting of a hypothetical dimer of trimers (Tanner *et al.*, 2001), the quaternary structure of the fungal enzyme remains to be unambiguously established.

Despite a failure to detect metal-dependence of turnover in earlier studies of native *Aspergillus niger* OxDC (Emiliani *et al.*, 1968), extensive comparisons of the deduced primary structures for oxalate oxidases and oxalate decarboxylases from a variety of sources suggest that OxDC contains two metal-binding sites per polypeptide. ICP-AE analysis of the purified enzyme from *Aspergillus niger* showed the sample to contain approximately 0.75 and 0.25 subunit-equivalents Mn and Cu, respectively. If bound Cu and Mn ions were both required for catalysis, it is anticipated that the maximum activity of the purified fungal enzyme would correspond to 3/16 of the theoretical V_{max}. While this is approximately the activity observed in the *Aspergillus niger* OxDC purified using the protocols described herein relative to that reported in previous studies of this enzyme (Emiliani *et al.*, 1968), the role of Cu in catalytic activity appears uncertain given that the recombinant bacterial OxDC has been demonstrated to contain only Mn (Tanner *et al.*, 2001). In any case, the metal content of the purified fungal OxDC is consistent with occupancy of no more than 50% of the potential active sites by either Cu or Mn.

Example 4 – Cloning, Expression and Purification of *Bacillus subtilis* Oxalate Decarboxylase.

Since the *Yvrk*-encoded protein had no known function at the time we initiated these studies, the upstream and downstream PCR primers were designed based on the published

sequence of the gene in the GenBank database, in order to clone the bacterial gene, and express and characterize its encoded protein. These primers were such that the *yvrK* coding sequence would be in-frame with the T7 control elements that are part of the pET-9a expression vector (Stratagene). An *Nde*I site was included at the N-terminal methionine, and a *BamH*I site after the termination codon of *yvrK*. *B. subtilis* 168 genomic DNA was purified from an overnight 5 mL culture using a Genomic DNA Miniprep kit (Qiagen). The *yvrK* sequence was amplified for 31 cycles (95 °C denaturation, 30s; 45 °C annealing, 30 s; 74 °C extension, 2 min). The resulting DNA was digested with *Nde*I and *BamH*I, then ligated into pET-9a digested similarly. Competent JM109 cells were transformed with the ligation mixture and with pET-9a as a control, and transformants selected on Luria-Bertani broth (LB) containing 30 μg/mL kanamycin (LBK). The resulting colonies were screened by *Nde*I-*BamH*I digestion to confirm the presence of a ~1153 bp insert, and the sequence of the cloned gene was checked by sequencing. A plasmid produced from pET-9a/*yvrK*:JM109 by standard alkaline lysis miniprep was used to transform the expression strain BL21(DE3), and the expression of the *Yvrk*-encoded protein was tested by inoculating 0.5 L of LBK supplemented with pET-9a/*yvrK*:BL21(DE3). The cells were grown at 37 °C and shaken at 200 r.p.m. When the cultures reached A_{600} of 2 they were heat shocked in water bath at 42 °C for 18 min before the addition of isopropyl thiogalactoside (IPTG) and $MnCl_2$ to final concentrations 1 and 5 mM respectively. The cells were harvested after 4 h of shaking by centrifugation (5,000 x g, 15 min, 4 °C). Pellets were resuspended in 50 mL lysis buffer (50 mM Tris/HCl pH 7; 10 μM $MnCl_2$) and sonicated for 30 s at 80 % power. After sonication, lysis pellets were separated from the crude extract by centrifugation (8000 rpm, 20 min, 4°C) and resuspended in 50 mL of extraction buffer containing 1 M sodium chloride, 0.1% Triton X-100, and 10 mM 2-mercaptoethanol. The mixture was stirred overnight at room temperature. Cell debris was removed by centrifugation and the supernatant was combined with the crude extract. This solution (100 mL) was diluted 10-fold before it was applied to a 2.5 x 30 cm DEAE-Sepharose Fast Flow (Sigma) column. This column was washed with 100 mL imidazole·HCl buffer (20 mM; pH 7.0 and 10 μM $MnCl_2$) and developed with a 500 mL M NaCl gradient (0 to 1M gradient). Ten mL fractions were collected and assayed for their ability to oxidize *o*-phenylenediamine, which is a side-reaction catalyzed by OxDC. Fractions exhibiting activity were pooled and solid $(NH_4)_2SO_4$ was added to a 70 % saturation. The precipitate was

removed by centrifugation (8,000 x g, 30 min, 4 °C) and redissolved in 200 ml imidazole·HCl buffer and the supernatant was loaded onto a phenyl-Sepharose Hi-Performance (Amersham Pharmacia Biotech) column. The column was washed with imidazole hydrochloride buffer (50 mM, pH 7.0, containing 10 μM $MnCl_2$) and developed with a 500 mL $(NH_4)_2SO_4$ gradient (1.7 to 0 M). The fractions were pooled as for the DEAE column and diluted 15-fold before they were loaded onto a Q-Sepharose Hi-Performance (Amersham Pharmacia Biotech) column. The protein was eluted with an imidazole hydrochloride buffer (50 mM, pH 7.0, containing 10 μM $MnCl_2$) and a 500 mL NaCl gradient (0 to 1 M) as for the DEAE column. Protein precipitated with 70 % ammonium sulfate was centrifuged and redissolved in 10 ml 20 mM hexamethylenetetramine·HCl pH 7. Ammonium sulfate was dialyzed out against 1L of the same amine buffer for 5 h at 4 °C. Protein solution was concentrated by centrifugal concentrator to final volume ~ 1 ml. Aliquots were flash-frozen in liquid nitrogen and stored at –80 °C. This procedure gave highly purified OxDC in yields of up to 30-40 mg/L with a specific activity of approximately 50 IU/mg.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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